

PCT

D INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 11/10, 11/04, 5/00, A61K 47/26	A1	(11) International Publication Number: WO 95/19430 (43) International Publication Date: 20 July 1995 (20.07.95)
(21) International Application Number: PCT/US95/00400 (22) International Filing Date: 12 January 1995 (12.01.95) (30) Priority Data: 08/181,269 13 January 1994 (13.01.94) US (71) Applicant (for all designated States except US): THE RO-GOSIN INSTITUTE [US/US]; 505 East 70th Street, New York, NY 10021 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): JAIN, Kanti [IN/US]; Apartment 40, 333 East 66th Street, New York, NY 10021 (US). RUBIN, Albert, L. [US/US]; 220 Allison Court, Engelwood, NJ 07631 (US). (74) Agent: HANSON, Norman, D.; Felfe & Lynch, 805 Third Avenue, New York, NY 10021 (US).	(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).  Published With international search report.	

(54) Title: MACROENCAPSULATED SECRETORY CELLS

## (57) Abstract

The present invention relates to macroencapsulation of secretory cells in a hydrophilic gel material, therapeutic methods employing the macroencapsulated secretory cells, and preserving the secretory cells by macroencapsulation.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## MACROENCAPSULATED SECRETORY CELLS

## Field of the Invention

The present invention relates to macroencapsulation of secretory cells in a hydrophilic gel material, therapeutic methods employing the macroencapsulated secretory cells, and preserving the secretory cells by macroencapsulation.

## Background of the Invention

Secretory cells are cells that are characterized by secreting biological products, such as, but not limited to, hormones (e.g., insulin), growth factors, cytokines, and so forth. Their role in biological processes is well known, and need not be set forth here. A number of diseases and pathological conditions are related to a failure of the secretory cells to work properly, such as a deficient production of the secretory products, e.g. hypothyroidism and cretin dwarfism, both due to thyroid hormone deficiency, hypophysial dwarfism due to pituitary growth hormone deficiency, Lesch-Hyhan Syndrome due to hypoxanthine-guanine phosphoribosyltransferase deficiency, fulminant hepatic failure due to the hepatotrophic factor deficiency, extracellular matrix disease due to chondrocyte deficiency, and insulin dependant diabetes due to insulin deficiency.

One approach to treat such conditions is to transplant the secretory cells into the patient. The transplanted material, in order to be clinically safe and effective, must (1) be non-immunogenic, non-thrombogenic, bio-stable, and completely non-toxic to cells and tissues of the host, (2) maintain cell viability for an extended period of time, (3) permit free passage of nutrients, secretagogues (a substance that stimulates secretion), and cell products, (4) facilitate surgical implantation and cell reseeded, and (5) be easily fixed in place and, likewise, removed.

Pancreatic islet transplantation to treat insulin-dependant diabetes has been the subject of renewed interest due to technological advances in isolating islets of Langerhans. By way of background, the human pancreas contains islets of Langerhans (hereinafter "pancreatic islets") that are scattered throughout the exocrine pancreas with some concentrations near the pancreatic ducts. The pancreatic islets, taken together, can be thought of as a single endocrine organ occupying around 1% of the volume of the pancreas. Within the pancreas, small islets (up to 160  $\mu$ m diameter) tend to distribute throughout the exocrine tissue. These small islets represent 75% of the islets in number

but only about 15% in volume. Islets greater than 250 $\mu$ m diameter constitute only 15% of the total number of islets but 60% of the volume. These islets are localized near larger ducts and blood vessels, and are not surrounded by acinar tissue. A human pancreas may contain over 1 million islets, and each islet typically consists of several thousand cells. Each islet is comprised of a central core of insulin producing beta cells (B-cells) and a surrounding mantle of glucagon containing alpha cells (A-cells), somatostatin secreting delta cells (D-cells) and pancreatic polypeptide containing cells (PP-cells). Insulin producing B-cells makeup the majority of the cells, and comprise up to about 80% of the islets in a human.

The clinical applications of pancreatic islet transplantation have been limited by the inability to prevent islet allograft-xenograft rejection, i.e., a rejection of the transplanted pancreatic islets due to the host's immune system attacking the transplanted pancreatic islets. To counteract the rejection, the pancreatic islets have been transplanted in combination with the administration of immunosuppressive agents.

Immunosuppressive therapy, however, has proved to be a double-edged sword; while reducing the risk of rejection, it impairs the body's overall immunological defenses. Various methods of protecting the transplanted tissue from the host immune response have been explored by many investigators. As discussed below, although temporary success has been reported (See Lacy, *Diabetes Reviews* 1 (1):76 (1993), effective long-term methods have yet to be achieved.

The five major approaches to protecting the transplanted tissue from the host's immune response all involve attempts to isolate the transplanted tissue from the host's immune system. The immunoisolation techniques used to date include: extravascular diffusion chambers, intravascular diffusion chambers, intravascular ultrafiltration chambers, microencapsulation, and macroencapsulation. All of these methods have failed, however, due to one or more of the following problems; a host fibrotic response to the implant material, instability of the implant material, limited nutrient diffusion across semi-permeable membranes, secretagogue and product permeability, and diffusion lag-time across semi-permeable membrane barriers.

For example, a microencapsulation procedure for enclosing viable cells, tissues, and other labile biological membranes within a semipermeable membrane was developed by Lim in 1978. (Lim, Research report to Damon Corporation (1978)). Lim used

microcapsules of alginate and poly L-lysine to encapsulate the islets of Langerhans. In 1980, the first successful *in vivo* application of this novel technique in diabetes research was reported ((Lim, et al., *Science* 210:908 (1980)). The implantation of these microencapsulated islets of Langerhans resulted in sustaining a euglycemic state in diabetic animals. Other investigators, however, repeating these experiments, found the alginate to cause a tissue reaction and were unable to reproduce Lim et al's results (Lamberti, et al., *Applied Biochemistry and Biotechnology* 10:101 (1984); Dupuy, et al., *Jour. Biomed. Material and Res.* 22:1061 (1988); Weber, et al., *Transplantation* 49:396 (1990); and Soon-Shiong, et al., *Transplantation Proceedings* 22:754 (1990)). The water solubility of these polymers is now considered to be responsible for the limited stability and biocompatibility of these microcapsules *in vivo* ((Dupuy, et al. *supra*, Weber, et al. *supra*, Soon-Shiong, et al., *supra*, and Smidsrod, *Faraday Discussion of Chemical Society* 57:263 (1974)).

Recently, Iwata et al., (Iwata, et al. *Jour. Biomedical Material and Res.* 26:967 (1992)) utilized agarose for microencapsulation of allogeneic pancreatic islets and discovered that it could be used as a medium for the preparation of microbeads. In their study, 1500-2000 islets were microencapsulated individually in 5% agarose and implanted into streptozotocin-induced diabetic mice. The graft survived for a long period of time, and the recipients maintained normoglycemia indefinitely.

Their method, however, suffers from a number of drawbacks. It is cumbersome and inaccurate. For example, many beads remain partially coated and several hundred beads of empty agarose form. Additional time is thus required to separate encapsulated islets from empty beads. Moreover, most of the implanted microbeads gather in the pelvic cavity, and a large number of islets are required in completely coated individual beads to achieve normoglycemia. Furthermore, the transplanted beads are difficult to retrieve, tend to be fragile, and will easily release islets upon slight damage.

A macroencapsulation procedure has also been tested. Macrocapsules of various different materials, such as poly-2-hydroxyethyl-methacrylate, poly vinylchloride-co-acrylic acid, and cellulose acetate were made for the immunoisolation of islets of Langerhans. (See Altman, et al., *Diabetes* 35:625 (1986); Altman, et al., *Transplantation American Society of Artificial Internal Organs* 30:382 (1984); Ronel, et al., *Jour. Biomedical Material Research* 17:855 (1983); Klomp, et al., *Jour. Biomedical*

*Material Research* 17:865-871 (1983)). In all these studies, only a transitory normalization of glycemia was achieved.

Archer et al., *Journal of Surgical Research*, 28:77 (1980), used acrylic copolymer hollow fiber to temporarily prevent rejection of islet xenografts. They reported long-term survival of dispersed neonatal murine pancreatic grafts in hollow fibers which were transplanted into diabetic hamsters. Recently Lacy et al., *Science* 254:1782-1784 (1991) confirmed their results, but found the euglycemic state to be a transient phase. They found that when the islets are injected into the fiber, they aggregate within the hollow tube and result in necrosis in the central portion of the islet masses. The central necrosis precluded prolongation of the graft. To solve this problem, they used alginate to disperse the islets in the fiber. Using this method they were able to achieve long-term graft survival. However, this experiment has not been extensively repeated. Therefore, the membrane's function as an islet transplantation medium in humans is questionable.

Thus, there exists a need for achieving secretory cell transplantation, and in particular, pancreatic islet allograft and xenograft survival without the use of chronic immunosuppressive agents.

The inventors have surprisingly discovered that macroencapsulating secretory cells in a hydrophilic gel material results in a functional, non-immunogenic, macrohead that can be transplanted into animals and can be stored for long lengths of time. The macroencapsulation of the secretory cells of the present invention provides a more effective and manageable technique for secretory cell transplantation. The macroencapsulation technique can also be used to macroencapsulate other biological agents, such as enzymes, microorganisms, trophic agents including recombinantly produced trophic agents, cytotoxic agents, and chemotherapeutic agents. The macroencapsulated biological agents can be administered to treat conditions known to respond to the biological agent.

### Summary of the Invention

It is therefore an object of the present invention to provide a secretory cell macrohead that can be transplanted into animals to treat conditions caused by an impaired functioning of the host's secretory cells.

5 It is a further object of this invention to provide a secretory cell macrohead that can be stored for long lengths of time.

In accomplishing these and other objects, there has been provided, in accordance with one aspect of the present invention a method of producing an agarose coated, agarose-collagen secretory cell macrohead; an agarose coated, gelfoam secretory cell  
10 macrohead; and a agarose coated, agarose secretory cell macrohead.

In another aspect of the invention, there is provided a method of treating a patient having a condition characterized by an insufficiency in a secretory cell product, comprising transplanting into said patient a therapeutically affective amount of secretory cell macroheads selected from the group consisting of agarose coated, agarose-collagen  
15 secretory cell macroheads; agarose coated, gelfoam secretory cell macroheads; and agarose coated, agarose secretory cell macroheads.

In yet a further aspect of the invention, there is provided a method for preserving secretory cells, comprising forming macroheads selected from the group consisting of agarose coated, agarose-collagen secretory cell macroheads; agarose coated, gelfoam  
20 secretory cell macroheads; and agarose coated, agarose secretory cell macroheads; and incubating said secretory cell macroheads.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments  
25 of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

### Brief Description of the Drawings

30 Figures 1 and 2 show agarose coated, collagen-agarose pancreatic islet macroheads.

Figure 3 shows the glucose levels of diabetic mice transplanted with agarose coated, collagen-agarose pancreatic islet macroheads.

Figure 4 shows the glucose levels of diabetic mice transplanted with agarose coated, gelfoam pancreatic islet macroheads.

5 Figure 5 shows the glucose levels of diabetic mice transplanted with agarose coated, agarose macroheads.

Figure 6 shows the glucose levels of diabetic mice transplanted with free pancreatic islets.

10 Figure 7 shows the glucose levels of diabetic mice transplanted with agarose coated, collagen-agarose macroheads and free pancreatic islets.

Figure 8 demonstrates a glucose tolerance test for normal mice, streptozotocin-induced diabetic mice, streptozotocin-induced diabetic mice receiving allotransplant in the kidney capsule "(KCT mice)", and streptozotocin-induced diabetic mice receiving agarose coated, agarose-collagen pancreatic islet macroheads; agarose coated, gelfoam pancreatic islet macroheads; and agarose coated, agarose pancreatic islet macroheads (collectively referred to as "head mice").

15

#### Detailed Description of the Invention

20 The present invention relates to macroencapsulation of biological agents, and preferably, secretory cells in a hydrophilic gel material, therapeutic methods employing the macroencapsulated biological agents, and preferably, secretory cells, and preserving the biological agents, preferably secretory cells by macroencapsulation. The hydrophilic gel material comprises agarose, and combinations of collagen-agarose and gelatin sponge-agarose. Gelatin sponge will hereinafter be referred to as gelfoam.

25 The term biological agent denotes a living organism and its products, e.g. proteins, enzymes, hormones, polypeptides, serum, antibodies, and antibiotics and also genetically engineered cells. Biological agents include enzymes, e.g., glucose oxidase, lactase complex, microorganisms, e.g., *Klebsiella aerogenes* for removal of ammonia and urea, trophic agents, including recombinantly produced trophic agents, e.g., recombinantly produced growth hormone, and cytotoxic agents.

30

The term secretory cell includes a pancreatic islet, although technically, a pancreatic islet is not a secretory cell, but mostly a cluster of secretory cells scattered



throughout the pancreas and comprising its endocrine portion. In humans, they are composed of at least four different types of secretory cells: alpha cells which secrete the hyperglycemic factor, glucagon; beta cells which are the most abundant (70% - 80%) and secrete insulin; delta cells which secrete somatostatin, and polypeptide cells which secrete polypeptide hormone.

As explained previously, transplanted material must be compatible with the host. Agarose has a long history of use in biological research, and its quality is well-controlled. Collagen is the most abundant protein in mammals, provides firm mechanical support and serves as the biological space for cell replication, differentiation, organogenesis, individual growth and wound repair. Collagen also has good biocompatibility. Gelfoam is non-immunogenic and has been used extensively in surgical procedures. It is also well-tolerated by secretory cells.

The biological agents, and preferably, secretory cells, are first isolated using procedures well known in the art. In a preferred embodiment, pancreatic islets are cultured at either 4°C, 24°C, or at 37°C before they are macroencapsulated. This method allows one to select only surviving islets after the isolation trauma. Also, the islets become less immunogenic resulting in the protection of macrobeads from fibrosis.

In one embodiment of the invention, a biological agent, preferably pancreatic islets, and more preferably about 50,000-700,000 pancreatic islets, are suspended in an aqueous solution of collagen, preferably about 0.5%-2% atellocollagen solution. Atellocollagen is obtained by treating collagen with pepsin, which removes antigenic telopeptides, responsible for intermolecular cross linkage of collagen. About 0.5%-5% of agarose, preferably about 1%, is then added to the suspended pancreatic islets to form pancreatic-islets suspended in a mixture of collagen and agarose. The mixture containing the pancreatic islets is then transformed into a semisolid bead using techniques well known in the art, preferably by dropping the mixture onto mineral oil or a Teflon® sheet. The semisolid bead is then transferred to an antibiotic medium, washed, and then incubated under standard conditions to polymerize the collagen, preferably at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, whereby a solid collagen-agarose macrobead is formed.

In another embodiment of the invention, a biological agent, preferably pancreatic islets, and more preferably about 50,000-700,000 pancreatic islets, are spread onto the

surface (3-5 cm) of a gelatin sponge. The gelatin sponge is then rolled into a sphere. Agarose, 3%-5%, is poured onto the sphere to form a bead.

5 In yet another embodiment of the invention, a biological agents, preferably pancreatic islets, and more preferably about 50,000-700,000 pancreatic islets, are placed in an agarose solution ranging from about 0.5%-5% agarose, preferably about 1% agarose. The mixture is then transformed into a macrobead by contacting the mixture to mineral oil or teflon. The bead is then transferred to an antibiotic medium, washed, and incubated overnight, preferably at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

10 In all the aforementioned embodiments, the macrobeads are uniformly coated with agarose, preferably by rolling the bead 3-4 times in a Teflon spoon containing about 500 - 2,000 ul of 5% - 10% agarose. Similarly, the term biological agent macrobeads, as used herein, denotes macroencapsulated biological agents in the form of a bead.

15 The macrobeads may be used as a vehicle to deliver the biological agent to the body where the agent will perform its known function. More than one type of biological agent may be encapsulated in one bead. For example, a macrobead can contain multiple enzymes, such as hemoglobin and glucose oxidase. Such a bead can be administered to remove bilirubin. These beads can be used either for oral administration of digestive enzymes (lactase complex) or for selective removal of undesirable amino acids from the body. Encapsulation of the enzymes will also prevent the degradation of the enzyme in the lumen. Furthermore, recombinant gene products can be safely delivered using encapsulation as the medium. K. aerogenes gene, for example, can be macroencapsulated in macrobeads for urea and ammonia removal. Where the biological agent is immunogenic to the host, the macrobead allows the administration of the biological agent without the use of immunosuppressant or with decreased amounts of immunosuppressant.

25 The secretory macrobeads may be used to treat conditions caused by an impaired functioning of the secretory cells of the subject, e.g. insulin dependant diabetes, growth factor deficiency disorder, and hormonal disorders, by transplanting the secretory cell macrobeads into the subject. The macrobeads may be inserted into the appropriate location for that particular treatment. For example, macrobeads containing hepatocytes can be implanted into the abdominal cavity to treat diseases related to liver non-function. A preferred application is transplanting 5-10 pancreatic islet macrobeads, each containing

30

50,000-700,000 pancreatic islets, into a patient to treat insulin-dependant diabetes. The macroheads can be inserted into the peritoneal cavity.

The secretory cell macroheads are transplanted into a patient in an amount sufficient to treat the condition. An amount adequate to accomplish this is defined as a "therapeutically effective amount" or "efficacious amount". Amounts effective for this use will depend upon the severity of the condition, the general state of the patient, the route of administration, the placement of macroheads, and whether the secretory cell macroheads are being administered in combination with other drugs.

The secretory macroheads can be used for allogeneic and xenogeneic transplantation in combination with immunosuppressants or preferably, without immunosuppressants. In a preferred embodiment, patients having chronic or acute insulin dependant diabetes are treated by xenotransplantating animal pancreatic islets, e.g. porcine, bovine, murine, rat, picin, or any other suitable species into the patient without the use of immunosuppressants. The secretory cell macroheads can also be administered in combination with other therapeutic agents, e.g. the commonly used triple drug therapy (cyclosporine, azathioprine, and hydrocortisone), rapamycin, deoxyspergualin or antibodies, to treat the condition.

The macroheads can also be used as a means to store the biological agents, and preferably secretory cells, for extended periods of time. To maintain the viability of the biological agents, and preferably secretory cells, the biological agents, and preferably secretory cell macroheads are incubated until they are transplanted in the animal.

When the secretory cells are pancreatic islets, the pancreatic islet macroheads are incubated at a temperature of 24°C or 37°C.

## EXAMPLES

### Example I

#### Pancreatic Islet Isolation

Pancreatic islets were isolated from rats by a modification of the method disclosed in Gotoh et al., *Transplantation* 40:437 (1985).

Collagenase solution (collagenase Type XI, Sigma Chemical, St. Louis, MO; 1mg/ml containing 2mg/ml of Sigma, Type V, bovine serum albumin and 1mg/ml  $\text{CaCl}_2$ ) was injected into the pancreas via the common bile duct. (Gotoh et al., *Transplantation*

40:437 (1985), Supra). The pancreas was removed and collected in a flask maintained on ice. Once pancreata from 4 rats had been collected, the flask was placed in a waterbath, at 38°C, for 30 minutes. The resulting digested tissue was washed 4 times in cold (8°C) HBSS (Hank's Balanced Salts Solution).

5 Undigested tissue, large lymph nodes, and other extraneous material were removed by repeated mobilization of the tissue, followed by removal of the supernatant. Purified islets were isolated on a discontinuous Ficoll gradient, consisting of 25%, 23%, 21%, and 11% Ficoll layers, prepared in Euro-Collins solution (Fresenius AG, Gluchen Steinweg, Homburg V.D.H.) and centrifuged at 2000 r.p.m. for 16 minutes. The islets were  
10 collected from the interface between 11% and 21% and the interface between 21% and 23% Ficoll layers. Islets from each fraction were pooled and washed four times in HBSS solution containing 10% fetal calf serum.

The pooled islets cells were then transferred to petri dishes containing RPMI complete medium, i.e., cold RPMI 1640 medium (GIBCO, Grand Island, NY),  
15 supplemented with 25 mM HEPES, heat-inactivated fetal bovine serum (10%), and antibiotic-antimycotic solution (1ml/100ml) which contains: 100ug/ml of penicillin, 100ug/ml of streptomycin sulfate, and 25ug/ml of amphotericin B. Any remaining non-islet acinar, vascular, ductular, or lymph node tissue was identified with the aid of a dissecting microscope, and carefully removed with a fine-tip sterile pipette. Final purity  
20 was assessed by staining the islet preparation with diphenylthiocarbazone.

After isolation, the islets were incubated in bacteriological plastic dishes (100 mm) containing 10ml of RPMI medium, at 37°C, in a humidified atmosphere having 5% CO<sub>2</sub>, for 4 days. The medium was changed every day, and the islets were then either directly  
25 transplanted or macroencapsulated.

## Example II

### A. Preparation of Agarose Coated, Agarose-Collagen-Pancreatic islet Macroheads

30 1000 pancreatic islets obtained by the method of Example I were washed four times in RPMI complete medium as described in Example I, less fetal calf serum. The pancreatic islets were then added to a tube containing 50μl of 1% atelocollagen solution in phosphate buffered saline, to suspend the pancreatic islets. 100μl of 1% low viscosity

agarose (Sigma Type XII) solution, prepared either in RPMI or in MEM (minimal essential medium), maintained at 60°C, was then added to the collagen-pancreatic islet suspension. The contents of the tube were then transferred immediately, as a single large drop; either onto sterilized mineral oil, maintained at room temperature, or onto a Teflon® sheet. After one minute, the drop became a semisolid macrobead which was then transferred to RPMI antibiotic medium, at 37°C. The macroheads were washed three times with the same medium to remove all oil. Finally, they were rinsed twice with complete medium (37°C) and incubated overnight, at 37°C, in a humidified atmosphere having 5% CO<sub>2</sub>. During this period, the collagen polymerized and the pancreatic islets rested on the collagen fiber.

The next day, the solid macroheads were transferred to a Teflon® spoon which contained approximately 1ml of 5% agarose in RPMI or in MEM medium. The solid macroheads were then rolled in this solution 2-3 times in order to uniformly coat them. Before the agarose solidified, the macroheads were transferred to mineral oil in a Teflon® dish to obtain smooth-surfaced macroheads. After 60 seconds, the macroheads were removed from mineral oil and washed 3 times with RPMI antibiotic medium, and then two times with RPMI complete medium. They were then incubated overnight, at 37°C, in a humidified atmosphere having 5% CO<sub>2</sub>. Agarose coated, agarose-collagen-pancreatic islet macroheads are shown in Figures 1 & 2.

B. Preparation of Agarose Coated,  
Gelatin Sponge Pancreatic Islet Macroheads

A small piece of gelatin sponge (gelfoam), 3mm<sup>2</sup>, was first soaked in RPMI complete medium. The medium was squeezed out and the gelfoam was allowed to rest for 1 minute. One thousand pancreatic islets, prepared according to Example I, were washed four times with RPMI antibiotic medium. They were then suspended in 10μl of RPMI antibiotic medium. They were transferred by a fine-tipped plastic pipette and spread onto the surface of the gelfoam. After 20 seconds, the gelfoam was rolled into a small sphere. 50μl of 5% agarose was poured onto the surface of the sphere to create an pancreatic islet macrobead.

In order to uniformly cover the macrobead with 5% agarose, 500μl of 5% agarose was added to the macrobead in a Teflon® spoon and was rolled 3-4 times. Before the agarose solidified, the macrobead was transferred to mineral oil, and the dish was rotated

to obtain a smooth surface on the macrohead. The macrohead was washed 3-4 times in RPMI antibiotic medium and then rinsed 2 times with RPMI complete medium. It was incubated overnight before being used for transplantation.

C. Preparation of Agarose Coated,  
Agarose Pancreatic Islet Macroheads

One thousand pancreatic islets obtained by the method of Example I were first washed 4 times in RPMI antibiotic medium. The pancreatic islets were transferred to a tube containing 50 $\mu$ l RPMI antibiotic medium and suspended thereon. 100 $\mu$ l of 1% agarose solution was then added to the tube. The entire contents of the tube was immediately transferred, as a single large drop, to either sterilized mineral oil or a teflon sheet. After 1 minute, the drop solidified to a macrohead. The macrohead was transferred to RPMI antibiotic medium, maintained at 37°C. The oil was then removed by washing the macrohead 3 times with the same medium, and then by rinsing 2 times with RPMI complete medium. The beads were incubated overnight at 37°C in a humidified atmosphere having 5% CO<sub>2</sub>.

The next day, these beads were transferred onto a Teflon® spoon containing 1ml of 5% agarose in either RPMI or in MEM medium. To uniformly coat the macrobeads with agarose, the beads were then gently rolled in agarose 2-3 times. They were then transferred to mineral oil, in a teflon dish, before the agarose solidified. After 60 seconds, the beads were removed from the mineral oil and washed 3 times in RPMI antibiotic medium and 2 times in RPMI complete medium. The beads were then incubated overnight.

**Example III -Transplantation of the Pancreatic Islet  
Macrobeads Into Mice**

A. Recipient Mice & Donor Rats

The mice used were male C57BL/6 and BALB/c strains. Recipient mice were made diabetic by a single i.v. injection of streptozotocin (170-200mg/kg).

Non-fasting plasma glucose levels were determined before the induction of diabetes. All blood sugar levels in the recipient mice were monitored via tail vein blood samples with an ExacTech Pen Sensor. Only those mice with serum glucose level >400mg/dl on the day of transplantation were used.

Wistar Furth rats were used as donors for xenotransplantation.

B. Xenotransplantation of Pancreatic islet Macroheads  
Into the Peritoneal Cavity

5 At the time of xenotransplantation, pancreatic islet macroheads of Example II(A), II(B), and II(C), respectively, were transferred gently to separate plates containing RPMI antibiotic medium. To remove all serum proteins, the medium was changed three times. Diabetic recipient mice were anesthetized with avertin. A midline incision was made to introduce a single pancreatic islet macrohead into the free peritoneal cavity. A two-layer  
10 closure of the incision was done with an absorbent suture. Control mice received either an empty macrohead i.p. (intraperitoneally), free pancreatic islets i.p., or an empty macrohead together with free donor pancreatic islets.

After transplantation, each recipient's blood glucose was checked daily or every  
15 other day until it reached the normal range; thereafter blood glucose was checked only 2-3 times every week. Transplants were considered technically successful if the serum glucose was <200mg/dl and remained there for consecutive bleedings. A transplant was considered to have been rejected if the serum glucose concentration rose above 200mg/dl after a period of transient normoglycemia. Transplants were considered to have failed or  
20 to have become 'primary nonfunctional' if the blood glucose never became normal (i.e., consistently remained >200mg/dl).

C. Intraperitoneal Glucose Tolerance Test

Approximately 70-84 days post-implantation, glucose tolerance tests were  
25 performed. Glucose solution (1.0g/kg body weight) was intraperitoneally injected into mice who had been fasting for 6 hours (9am-3pm). Both pre- and post-injection (0, 30, 60, and 120 minutes), blood samples were taken to determine plasma glucose levels using the ExacTech Pen Sensors.

For comparison, glucose tolerance tests were performed on normal C57BL/6 and  
30 BALB/c mice, on streptozotocin induced C57BL/6 and BALB/c mice in which no pancreatic islets had been transplanted, and on streptozotocin-induced diabetic BALB/c mice in which free pancreatic islets had been transplanted into the kidney capsule ("KCT" mice).

Control experiments were conducted to ensure that the euglycemic state in diabetic mice was being achieved via the macroencapsulated pancreatic islets and not the macrobeads themselves. Empty agarose coated, agarose-collagen macrobeads and agarose coated, gelfoam macrobeads were, therefore, prepared in the same manner as the beads of Examples II(A) and (B).

D. Results of the Intraperitoneal Xenotransplantation and Glucose Tolerance Test

Upon implantation of pancreatic islet macrobeads, the changes observed in the non-fasting plasma glucose level of STZ-diabetic streptozotocin induced C57BL/6 mice are shown in Figures 3 & 4. The recipients of agarose coated, agarose-collagen pancreatic islet macrobeads and agarose coated, gelfoam pancreatic islet macrobeads maintained a normoglycemic state for more than 60 days and, during this period, the body weight of these mice increased an average of 3 grams. When agarose covered, agarose pancreatic islet macrobeads were transplanted, 2 of 6 animals became normoglycemic after 21-33 days post transplantation (Figure 5) and remained euglycemic thereafter. All other transplanted animals failed to achieve a euglycemic state. Empty macrobeads (n=6) did not affect blood glucose.

When free pancreatic islets were transplanted intraperitoneally, 6 of 7 transplanted animals became normoglycemic 1 day after transplantation; however, they maintained this state for only 3-10 days (Figure 6). When free pancreatic islets were transplanted with empty beads made of agarose coated, agarose-collagen macrobeads or agarose coated, gelfoam macrobeads, all the animals became normoglycemic within 24 hours and remained so for more than 12 days (Figure 7). Subsequently, all animals became hyperglycemic. Animals which contained empty macrobeads excited no tissue reaction for the 90 days they were followed.

The results obtained after performing the Glucose Tolerance Tests are presented in Figure 8. In normal BALB/c and C57BL/6 mice and "KCT" mice, plasma glucose peaked at 30 minutes and returned to baseline levels by 120 minutes.

Similar results were obtained when macroencapsulated pancreatic islets and non-encapsulated pancreatic islets transplanted in the kidney capsule were tested.



The results of these experiments demonstrate that the agarose coated, agarose-collagen islet macroheads; agarose coated, agarose-gelfoam pancreatic islet macroheads; and agarose coated, agarose islet macroheads display the properties required for a hybrid artificial organ. Although all three types successfully secrete insulin, agarose coated, agarose-collagen and agarose coated, agarose-gelfoam macroheads are more suitable as biohybrid artificial organs due to the uniformity of results obtained in the minimum number of transplanted animals. Moreover, all the three types of beads showed no adverse effects. The macroheads remained free in the peritoneum showing neither tissue reaction, nor any adhesion to any organ. Thus, these biohybrid pancreatic islets perform their function as efficiently in the macroencapsulated beads as in their natural habitat, the pancreas.

In all of the mice, plasma glucose peaked at 30 minutes and returned to baseline levels by 120 minutes.

#### Example IV

##### Extended Storage Life of Pancreatic islets

Macroencapsulated beads prepared according to Examples I(A), (B), and (C), which were incubated for 4 weeks at 37C in complete RPMI medium, were tested for their long-term preservation properties *in vivo* and *in vitro*. It was found that the macroencapsulated pancreatic islets which were incubated for 4 weeks were functionally similar to those which were incubated for 1 day.

This example demonstrates that the method of macroencapsulation according to the present invention can be used for secretory cell preservation, and preferably, pancreatic islet preservation.

*What Is Claimed Is:*

1. A method of producing an agarose coated, agarose-collagen secretory cell macrobead comprising;

- (a) suspending secretory cells in a solution containing collagen,
- 5 (b) adding agarose to said suspended secretory cells of step (a) to form secretory cells suspended in a mixture of agarose and collagen,
- (c) forming a collagen-agarose semisolid macrobead from said suspended secretory cells of step (b),
- 10 (d) treating said collagen-agarose semisolid macrobead of step (c) to polymerize collagen contained in said semisolid macrobead, whereby a solid collagen-agarose macrobead is formed,
- (e) coating said solid macrobead of step (d) with agarose to obtain an agarose coated, agarose-collagen secretory cell macrobead.

15 2. The method of claim 1, wherein step (e) comprises rolling said solid macrobead of step (d) in 5% agarose, contacting said rolled solid macrobead to mineral oil, and washing said rolled macrobead to obtain said agarose coated, agarose-collagen secretory cell macrobead.

3. The method of claim 1, wherein said secretory cells are pancreatic islets.

20 4. The method of claim 3, wherein said pancreatic islets are human pancreatic islets.

5. The method of claim 3, wherein said pancreatic islets are bovine pancreatic islets.

25 6. The method of claim 3, wherein said pancreatic islets are porcine pancreatic islets.

7. The method of claim 3, wherein said macrobead contains from about 50,000 to about 700,000 pancreatic islets.

8. A method of producing an agarose coated, gelfoam secretory cell macrobead comprising;

- (a) suspending secretory cells on gelfoam,
- 30 (b) rolling said gelfoam containing said suspended secretory cells into a sphere,

(c) coating said sphere with agarose to obtain an agarose coated, gelfoam secretory cell macrobead.

9. The method of claim 8, wherein step (c) comprises:

- (1) pouring agarose on the surface of said sphere to form a macrobead,
- (2) rolling said macrobead in 5% agarose,
- (3) contacting said rolled macrobead produced in step (2) to mineral oil,
- (4) and washing the macrobead of step (3) to form said agarose coated, gelfoam secretory cell macrobead.

10. The method of claim 8 wherein said secretory cells are pancreatic islets.

11. The method of claim 10, wherein said pancreatic islets are human pancreatic islets.

12. The method of claim 10, wherein said pancreatic islets are bovine pancreatic islets.

13. The method of claim 10, wherein said pancreatic islets are porcine pancreatic islets.

14. The method of claim 10, wherein said macrobead contains from about 50,000 to about 700,000 pancreatic islets.

15. A method of producing an agarose coated, agarose secretory cell macrobead comprising;

- (a) suspending secretory cells in agarose,
- (b) forming a macrobead from said suspended secretory cells of step (a),
- (c) incubating said macrobead of step (b) in humidified air,
- (d) coating said macrobead of step (c) with agarose to form an agarose coated, agarose secretory cell macrobead.

16. The method of claim 13, wherein step (e) comprises rolling said solid macrobead of step (c) in 5% agarose, contacting said rolled solid macrobead to mineral oil, and washing said rolled macrobead to form said agarose coated, agarose secretory cell macrobead.

17. The method of claim 15 wherein said secretory cells are pancreatic islets.

18. The method of claim 17, wherein said pancreatic islets are human pancreatic islets.

19. The method of claim 17, wherein said pancreatic islets are bovine pancreatic islets.

20. The method of claim 17, wherein said pancreatic islets are porcine pancreatic islets.

5 21. The method of claim 17, wherein said macrobead contains from about 50,000 to about 700,000 pancreatic islets.

22. An agarose coated, agarose-collagen secretory cell macrobead.

23. The agarose coated, agarose-collagen secretory cell macrobead of claim 22, wherein said secretory cell is a pancreatic islet.

10 24. The agarose coated, agarose-collagen secretory cell macrobead of claim 23, wherein said pancreatic islet is a human pancreatic islet.

25. The agarose coated, agarose-collagen secretory cell macrobead of claim 23, wherein said pancreatic islet is a bovine pancreatic islet.

15 26. The agarose coated, agarose-collagen secretory cell macrobead of claim 23, wherein said pancreatic islet is a porcine pancreatic islet.

27. An agarose coated, gelfoam secretory cell macrobead.

28. The agarose coated, gelfoam secretory cell macrobead according to claim 27, wherein said secretory cell is a pancreatic islet.

20 29. The agarose coated, agarose-collagen secretory cell macrobead of claim 28, wherein said pancreatic islet is a human pancreatic islet.

30. The agarose coated, agarose-collagen secretory cell macrobead of claim 28, wherein said pancreatic islet is a bovine pancreatic islet.

31. The agarose coated, agarose-collagen secretory cell macrobead of claim 28, wherein said pancreatic islet is a porcine pancreatic islet.

25 32. An agarose coated, agarose secretory cell macrobead.

33. An agarose coated, agarose secretory cell macrobead according to claim 32, wherein said secretory cell is a pancreatic islet.

34. The agarose coated, agarose-collagen secretory cell macrobead of claim 33, wherein said pancreatic islet is a human pancreatic islet.

30 35. The agarose coated, agarose-collagen secretory cell macrobead of claim 33, wherein said pancreatic islet is a bovine pancreatic islet.

36. The agarose coated, agarose-collagen secretory cell macrobead of claim 33, wherein said pancreatic islet is a porcine pancreatic islet.

37. A method of treating a patient having a condition caused by an impaired functioning of secretory cells:

5 transplanting into said patient a therapeutically affective amount of secretory cell macrobeads selected from the group consisting of agarose coated, agarose-collagen secretory cell macrobeads; agarose coated, gelfoam secretory cell macrobeads; and agarose coated, agarose secretory cell macrobeads.

10 38. The method of claim 37, wherein said condition is insulin dependent diabetes.

39. The method of claim 38, wherein said secretory cell is a pancreatic islet.

40. The method of claim 39, wherein said pancreatic islet is a human pancreatic islet.

15 41. The method of claim 39, wherein said pancreatic islet is a porcine pancreatic islet.

42. The method of claim 39, wherein said pancreatic islet is a bovine pancreatic islet.

43. The method of claim 39, wherein said secretory cell macrobeads are placed in the intraperitoneal cavity.

20 44. The method of claim 39, wherein about 5 to about 10 macrobeads are inserted, each macrobead containing from about 50,000 to about 700,000 pancreatic islets.

45. The method of claim 39, wherein said secretory cell macrobeads are agarose coated, agarose-collagen secretory cell containing macrobeads.

25 46. The method of claim 39, wherein said secretory cell macrobeads are agarose coated, gelfoam secretory cell macrobeads.

47. The method of claim 39, wherein said secretory cell macrobeads are agarose coated, agarose secretory cell macrobeads.

48. A method for preserving secretory cells, comprising:

30 (a) forming macrobeads selected from the group consisting of agarose coated, agarose-collagen secretory cell macrobeads; agarose coated, gelfoam

secretory cell macroheads; and agarose coated, agarose secretory cell macroheads; and

(b) incubating said secretory cell macroheads.

5 49. The method according to claim 48, wherein said secretory cell is a pancreatic islet.

50. The method according to claim 49, wherein said pancreatic islet is incubated at 24°C or 37°C.

FIG. 1

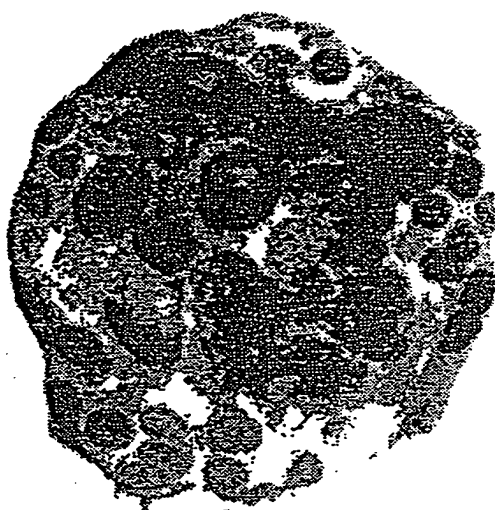


FIG. 2

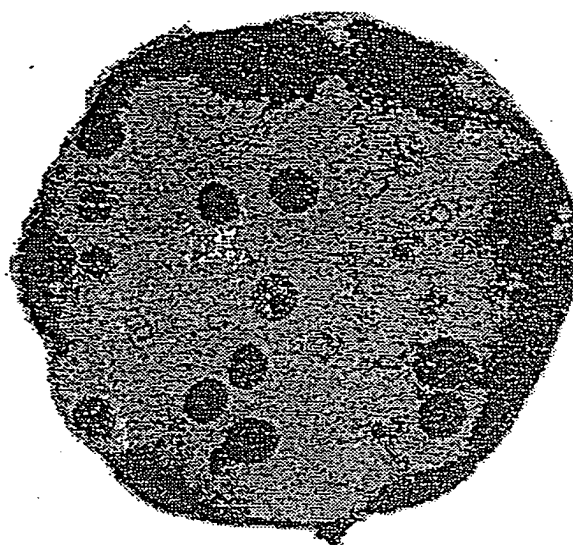




FIG. 3

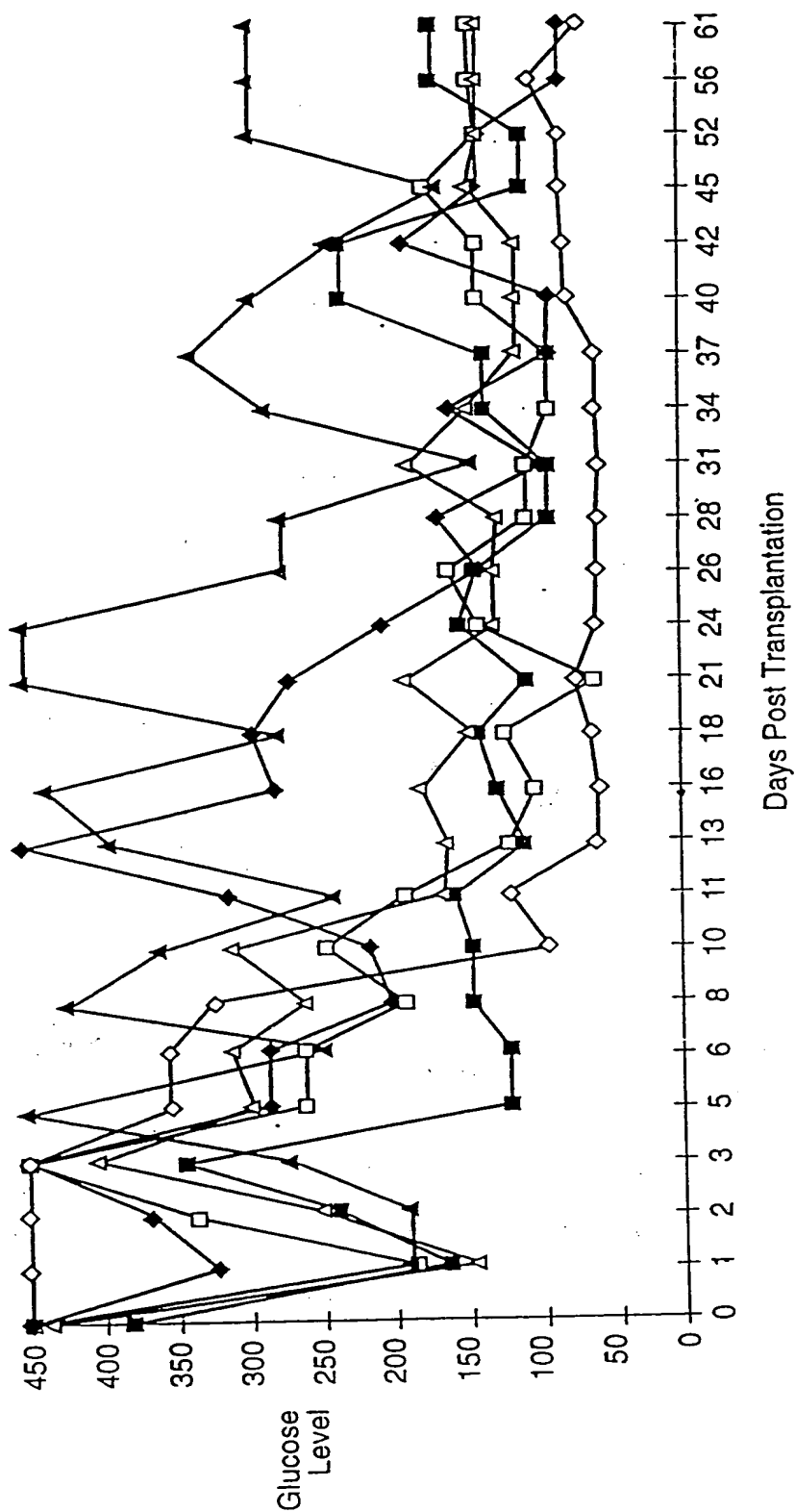


FIG. 4

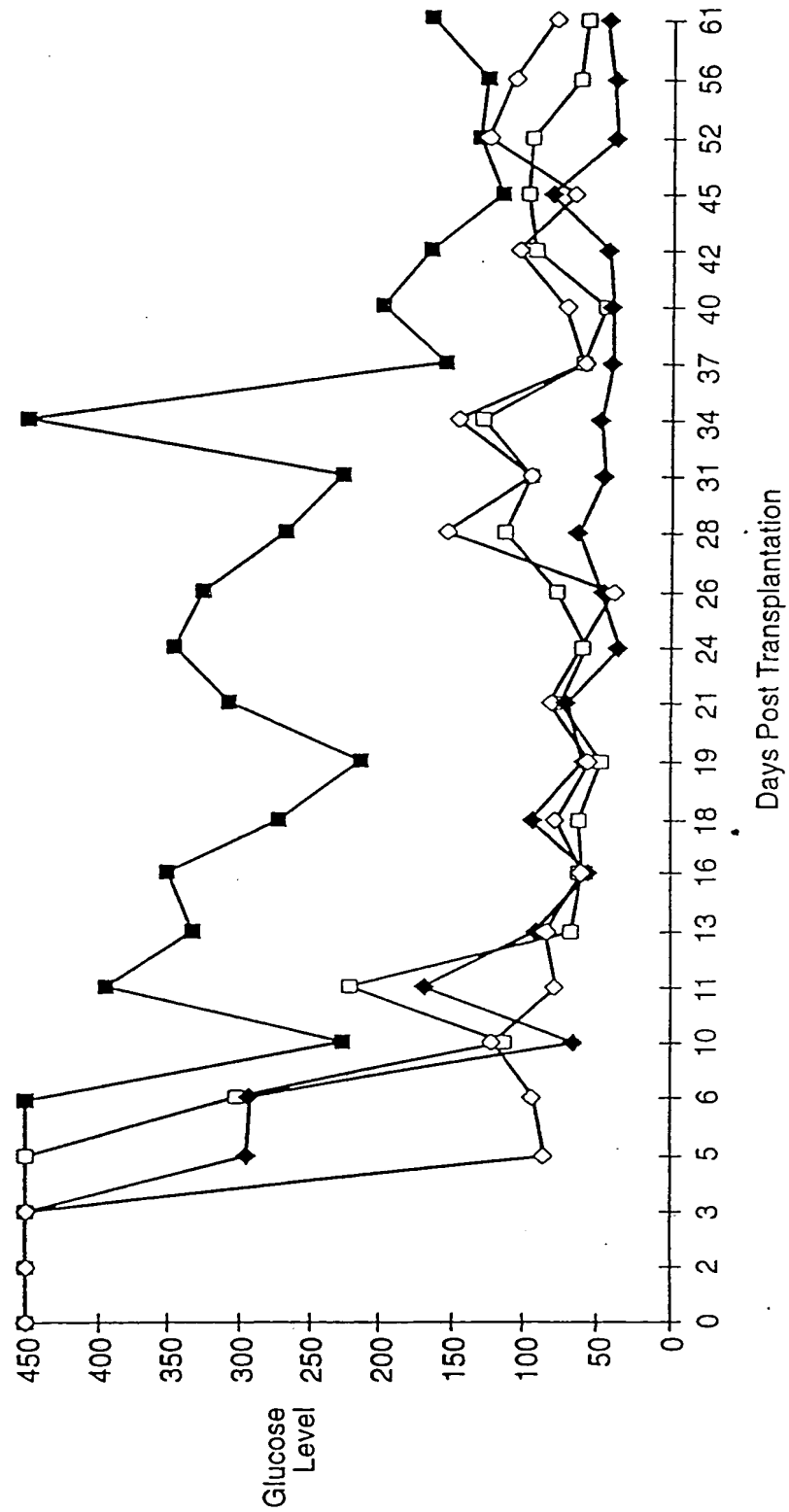
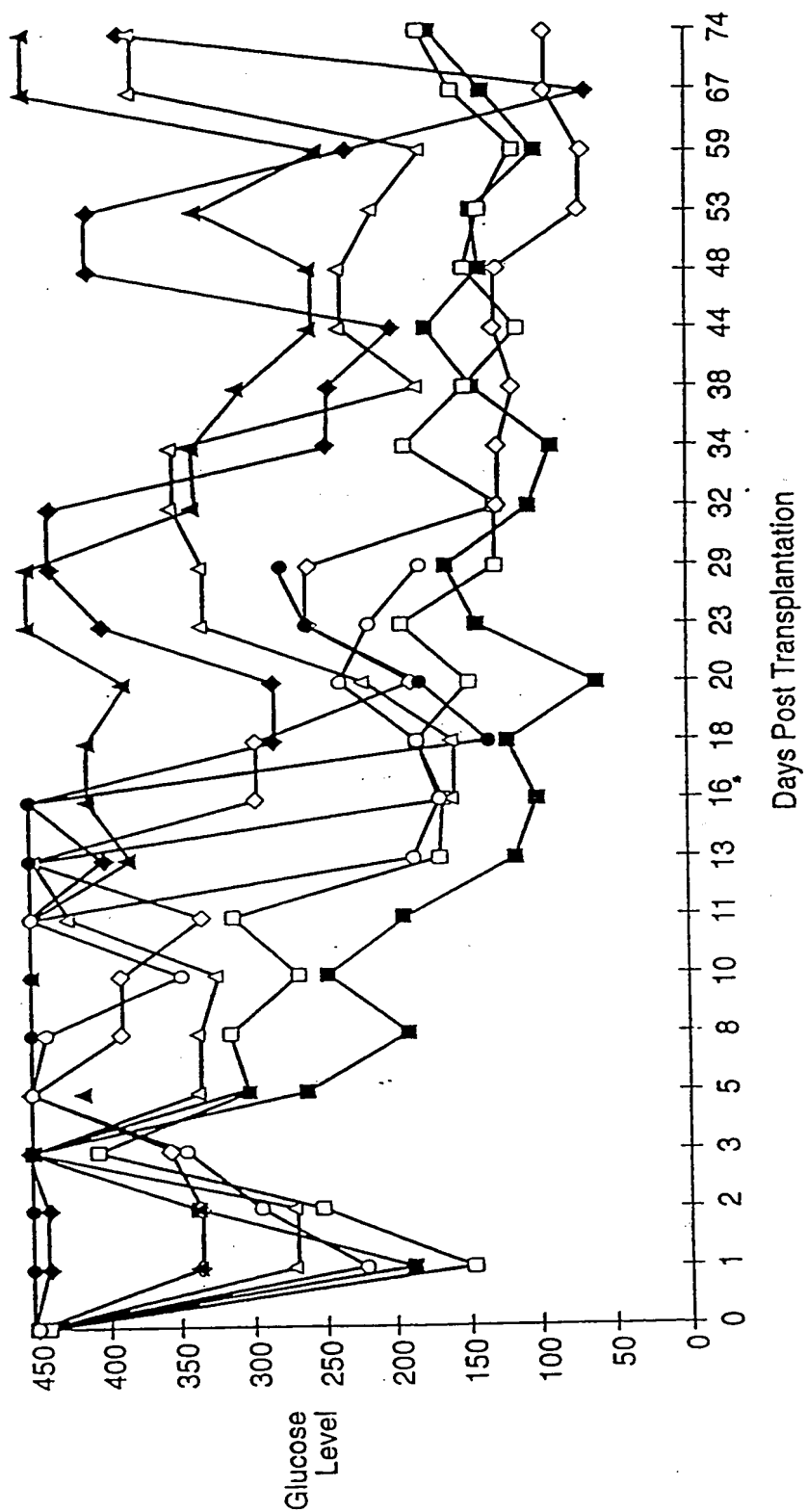


FIG. 5.



6/8

FIG. 6

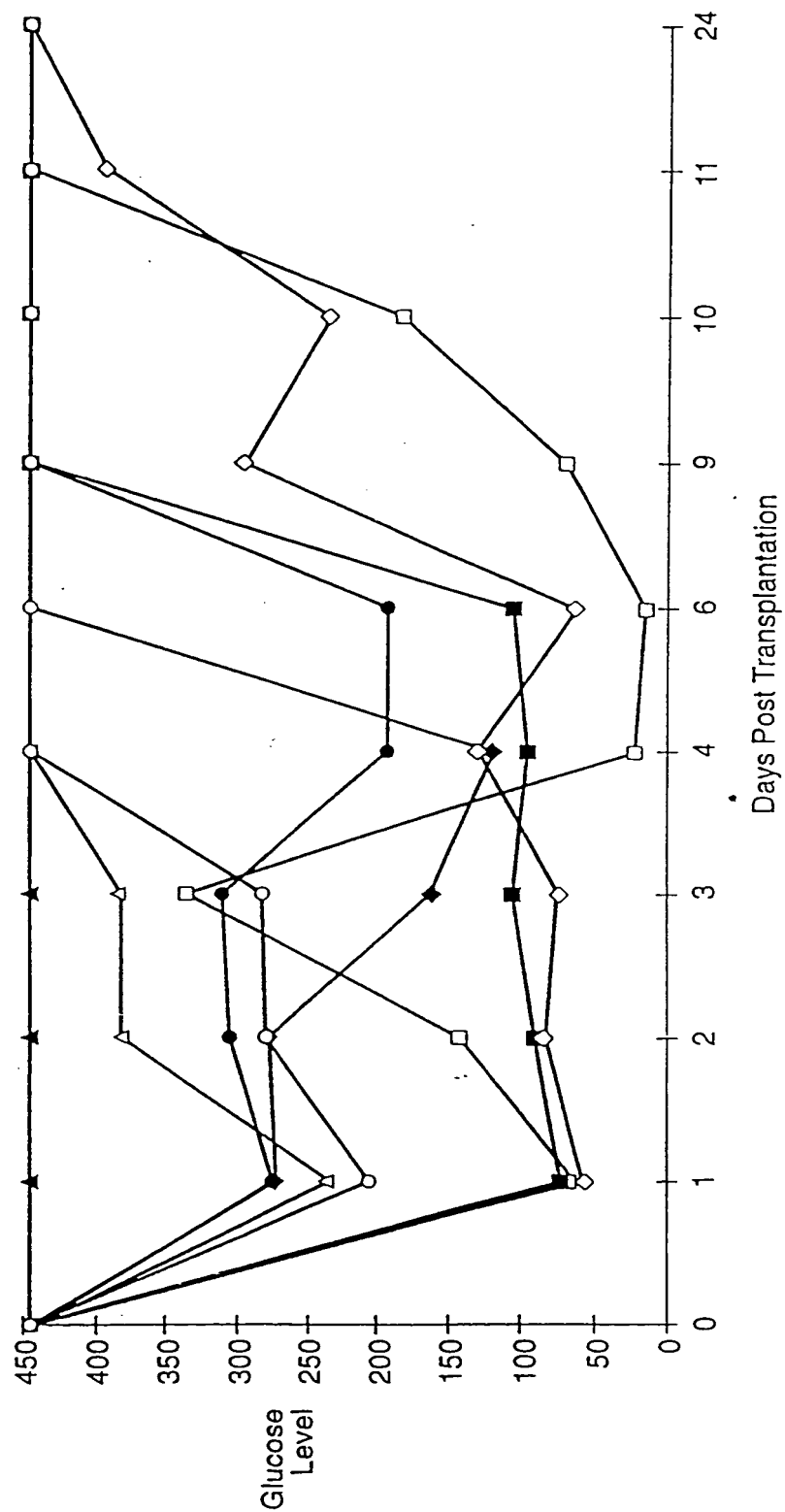
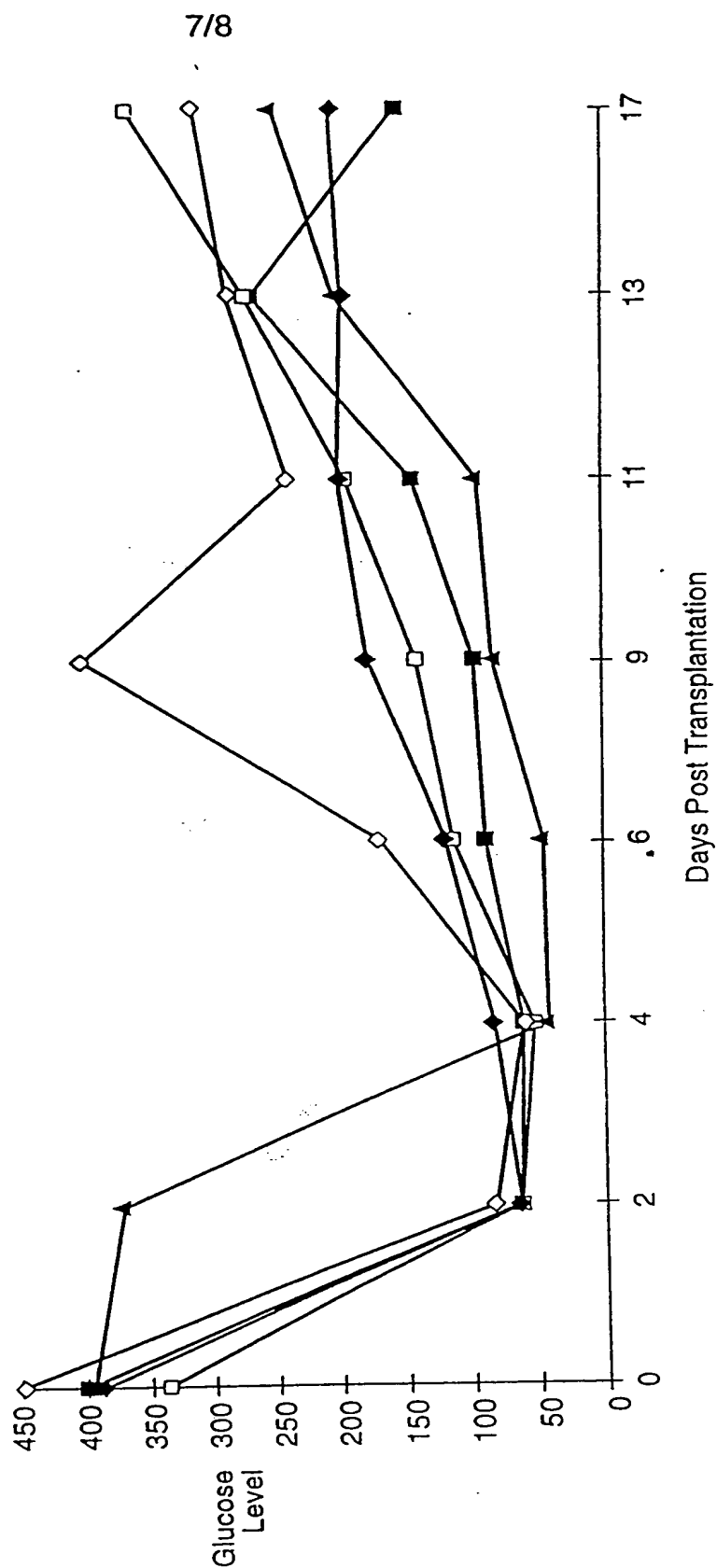
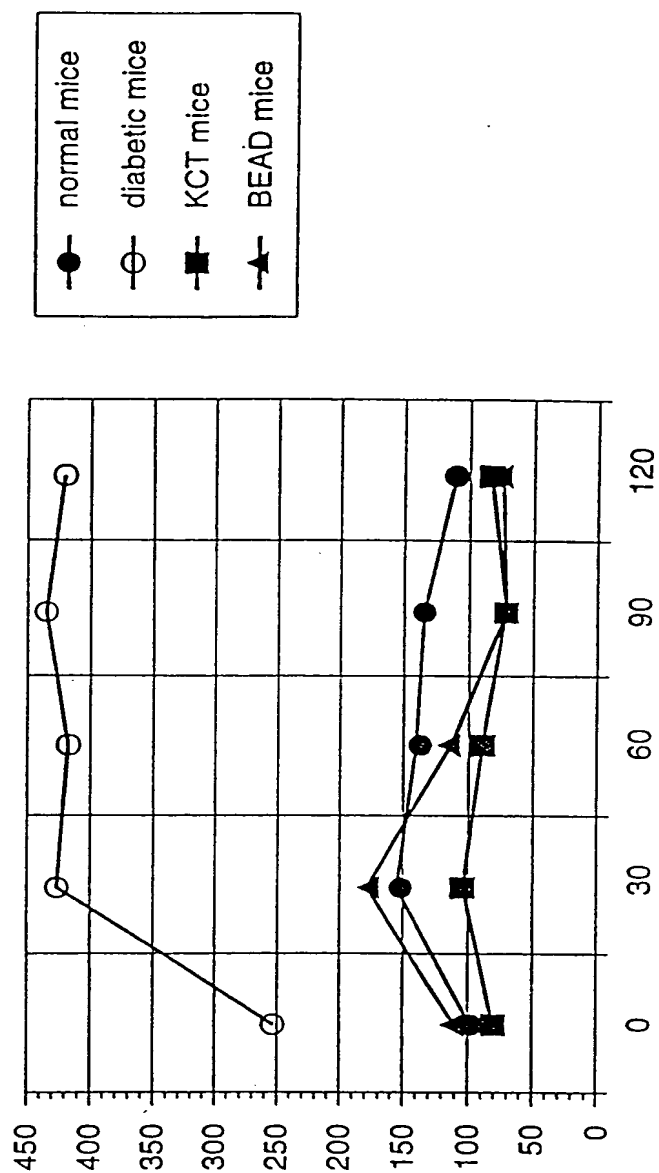


FIG. 7



8/8

FIG. 8



## INTERNATIONAL SEARCH REPORT

International application No.

CT/US95/00400

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 11/10, 11/04, 5/00; A61K 47/26

US CL :435/178, 182, 240.22; 424/93.7

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/178, 177, 182, 240.22; 424/93.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

search terms: agarose, encapsulation, coated, secretory cells, bovine pancreatic islets, porcine pancreatic islets, collagen

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,053,332 (COOK ET AL.) 01 October 1991, entire document.	1-7, 15-26, 32-45, 47-50
Y	US, A, 4,663,286 (TSANG ET AL.) 05 May 1987, entire document.	1-50
Y	US, A, 4,971,833 (LARSSON ET AL.) 20 November 1990, entire document.	1-50
Y	US, A, 4,673,566 (GOOSEN ET AL.) 16 June 1987, entire document.	1-50
Y	US, A, 4,647,536 (MOSBACH ET AL.) 03 March 1987, entire document.	1-7, 22-26, 45

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

11 APRIL 1995

Date of mailing of the international search report

17 APR 1995

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DAVID M. NAFF

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/00400

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FEBS Letters, Volume 122, Number 2, issued December 1980, Brodelius et al., "Entrapment of Plant Cells In Different Matrices", pages 312-316, entire document.	1-7, 22-26, 45
Y	US, A, 4,798,786 (TICE ET AL.) 17 January 1989, entire document.	8-14, 27-31, 46